Effect of dietary bile extracts on serum response of astaxanthin in rainbow trout (*Oncorhynchus mykiss*): a preliminary study

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Abstract

Effects of porcine bile extracts added at three different dietary concentrations 0, 10 and 20 g kg^{-1} were studied on astaxanthin serum concentration in rainbow trout (mean weight 200 ± 7 g). Astaxanthin from micro-algae *Haematococcus pluvialis* and synthetic astaxanthin (CAROPHYLL® pink) were incorporated in diets of rainbow trout at a rate of 100 mg astaxanthin kg^{-1} of feed. Fish were hand fed twice a day. After 5 days of feeding there was a significant effect of the pigment source on the ratio (total blood astaxanthin per unit body weight to cumulative astaxanthin intake per unit body weight). Trout receiving synthetic astaxanthin showed a significantly ($P < 0.05$) higher ratio than trout fed algal astaxanthin. Increasing dietary bile extract did not lead to produce any effect on this ratio. The power of the statistical analysis is discussed. Therefore, the interaction (pigment source × dietary bile concentration) showed no more effect.

KEY WORDS: astaxanthin, bile extracts, *Haematococcus pluvialis*, pigmentation, serum, trout

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Introduction

Astaxanthin is widespread in nature and is the main pigment in the marine environment. As astaxanthin cannot be synthesized *de novo* by fish, especially salmonids, its concentration in the flesh depends on adequate ingestion and efficient intestinal absorption from exogenous sources. In the wild, fish obtain astaxanthin from prey while in intensive culture salmonid feed must be supplemented with astaxanthin to enhance their natural pigmentation (Choubert *et al.* 1995; Choubert & Storebakken 1996).

Haematococcus pluvialis is a fresh water unicellular green micro-alga which form cysts and accumulate massive amounts of astaxanthin in its cytoplasm under certain stress conditions (Goodwin & Jamirkon 1954; Borowitzka *et al.* 1991; Boussiba *et al.* 1992) and its use for pigmentation in aquaculture has been proposed for many years. However, the effect of feeding algal astaxanthin has been reported in the past showing a lower trout flesh pigmentation when comparing with synthetic astaxanthin (Sommer *et al.* 1991, 1992; Choubert & Henrich 1993; White *et al.* 2002).

Carotenoids are lipid-soluble compounds. Their absorption into the intestinal mucosal cells is considered to occur by passive diffusion (Deming & Erdman 1999). Carotenoid absorption requires solubilization into mixed micellar solutions before efficient absorption can occur (El-Gorab *et al.* 1975). Mixed micelles are formed from bile salts and lipolytic products of dietary fat during intestinal absorption (Deming & Erdman 1999). The bile by its detergent properties enhances emulsion formation of fat and then assists in the digestion and absorption of carotenoid content herein (Gagnon & Dawson 1968; Klaüi & Bauernfeind 1981). Therefore, one can expect an enhancement in carotenoid absorption caused by bile salt supplementation into the diets.

The goal of this study was to evaluate the effect of incremental increases in the diets porcine bile concentrations on astaxanthin concentration in the serum of fish fed either synthetic astaxanthin or algal astaxanthin from *Haematococcus pluvialis*.

Materials and methods

Fish and feeding management

The experiment took place at the INRA recirculating water system at Saint Pée-sur-Nivelle (Pyrénées Atlantiques Department, France). Rainbow trout *Oncorhynchus mykiss* from the same parental stock were provided by the INRA experimental fish farm of Donzacq (Landes Department, France). Seventy two fish with a mean weight of 200 ± 7 g were randomly divided in different groups in square tanks (300 L) set in parallel rows supplied with a recirculated water maintained at a constant temperature (17 ± 1 °C). Prior to introduction to the experimental diets and during the acclimatization period, fish were fed for 15 days a commercial non-pigmented trout feed (Trouw France, Fontaine-les-Vervins, France). Then fish were fed the six experimental diets in duplicate for 5 days. Fish were hand fed twice a day at a rate of 2.5% body weight day^{-1} (BWD) and complete feed ingestion was assessed visually.

Experimental diets

Six test diets with the same basal composition (Table 1) were supplemented with two different astaxanthin sources at a level of 100 mg astaxanthin kg^{-1} of feed and three different levels, 0, 10 and 20 g kg^{-1} of porcine bile extract (B8631; Sigma-Aldrich Quimica s.a., Lisboa, Portugal). Two carotenoid sources were used: algae *Haematococcus pluvialis* strain CCAP-34/7 (Culture Collection of Algae and Protozoa, Windermere, UK) cultivated under the same conditions as described by Mendes-Pinto *et al.* (2001) and commercial beadlets of 8% (w/w) astaxanthin content (CAROPHYLL® Pink; F. Hoffmann-La Roche, Basel, Switzerland). Algae were mechanically ground in a ball grinder (grinder Dangoumeau; Prolabo, Fontenay-sous-bois, France) for 5 min prior to diet incorporation and the cell wall disruption was assessed by optical microscopy (BioMed, Leitz, Westlar, Germany).

Diets were pelleted using a steamless pelleting machine (Regina Supernova, Italy) through a 4.5 mm dye. Pellets were allowed to dry at 38 °C for 22 h and were stored at +4 °C prior to use. Proximate composition of experimental diets are given in Table 1.

Sampling and analytical methods

At the end of the experiment and 30 min after the last meal, fish blood was collected from the caudal artery with a 2 mL non-heparinized disposable syringes fitted with 0.6 × 25 mm

Table 1 Formulas, ingredients and chemical composition of the six experimental feeds (ALG, AST: diets containing algae or synthetic astaxanthin respectively with 0, 10, 20 g kg^{-1} porcine bile)

Diet label	0-ALG	1-ALG	2-ALG	0-AST	1-AST	2-AST
Feed ingredients (g kg^{-1})						
Fish meal	580	580	580	580	580	580
Gelatinized wheat starch	135	135	135	135	135	135
Crude wheat starch	240	230	220	240	230	220
Fish oil	15	15	15	15	15	15
Vitamin mix[1]	10	10	10	10	10	10
Mineral mix[2]	10	10	10	10	10	10
Sodium alginate	10	10	10	10	10	10
Porcine bile[3]	0	10	20	0	10	20
Synthetic astaxanthin[4]				+	+	+
Algae[5]	+	+	+			
Feed chemical composition						
Dry matter (DM) (g kg^{-1})	912.0	857.6	894.6	831.4	849.6	880.7
Total lipids (g kg^{-1} DM)	79.0	88.3	83.7	84.8	86.0	90.4
Astaxanthin (mg kg^{-1} DM)	86.1	87.6	91.3	102.4	104.2	105.6

[1] INRA 762. Vitamin mix contained the following diluted in cellulose (g kg^{-1} mix): vitamin A (500 000 IU g^{-1}), 1.5; vitamin D3 (100 000 IU g^{-1}), 1.5; vitamin E (500 IU g^{-1}), 6; vitamin K, 0.25; thiamin, 0.75; riboflavin, 1.5; pyridoxine, 0.75; nicotinic acid, 8.75; vitamin C, 25; folic acid, 0.25; vitamin B12 (1000 mg kg^{-1}), 2.5; inositol, 50; biotin (2 mg kg^{-1}), 6.25; calcium pantothenate, 2.5; choline (50 mg kg^{-1}), 200.

[2] INRA 763. Mineral mix contained the following ingredients (g kg^{-1} mix): calcium carbonate, 215; magnesium hydroxyde, 124; KCl, 90; ferric citrate, 20; KI, 0.4; NaCl, 40; calcium hydrogen phosphate ($CaHPO_4$), 500; copper sulphate, 3; zinc sulphate, 4; cobalt sulphate, 0.2; manganese sulphate, 3.

[3] Sigma-Aldrich Quimica s.a., Lisboa, Portugal (product no. B8631).

[4] CAROPHYLL® pink, F. Hoffmann-La Roche, Basel, Switzerland. Supplementation level: 100 mg astaxanthin kg^{-1} of feed.

[5] Supplementation level: 100 mg astaxanthin kg^{-1} of feed.

disposable needles (Becton Dickinson France, Le Pont de Claix, France). Approximately 2.0 mL of blood sample per fish was held overnight at +4 °C for clotting. Serum was removed after sample centrifugation at $2000 \times g$ (Model T52.1, MLW, Engelsdorf, Germany) for 5 min.

Serum (0.5 mL) was vortexed with 1 mL ethanol (95 °C) for 30 s, followed by addition of 1 mL *n*-hexane (both from Carlo Erba, Rodano, Italy); the mixture was vortexed for 1 min. The *n*-hexane was separated by centrifugation at $2000 \times g$ for 5 min. The process was repeated twice to complete astaxanthin extraction. Absorbance was measured at 480 nm in *n*-hexane using a UV–Visible spectrophotometer (UV-160A; Shimadzu, Tokyo, Japan). Astaxanthin concentration was calculated using a specific extinction coefficient of 2100 in *n*-hexane (Weber 1988).

For carotenoid extraction, micro-algal biomass was prior mechanically ground during 5 min (grinder Dangoumeau). Then carotenoids and lipids were extracted using the method of Folch *et al*. (1957). Determination of total carotenoids content was made by UV–Visible spectrophotometry. Extracts were resuspended in 20 mL dichloromethane and the specific extinction coefficient used was 2100 in dichloromethane (Weber 1988). All calculations were made on a dry matter basis. Algal carotenoid separation was made by TLC on pre-coated silica gel H60 TLC plates 20 × 20 cm (Merck, Darmstad, Germany) using as solvent system acetone: *n*-hexane 3 : 7 (v/v) (Kobayashi *et al*. 1991) at room temperature. In a diffused light different TLC bands corresponding to free, mono and diester astaxanthin (according to their R_f values) were scrapped off, eluted with acetone and vacuum filtered. Each band extract was dried under vacuum in a rotary evaporator (RE-121 mod.; Büchi Laboratoriums Technik, Flawil, Switzerland) and resuspended in dichloromethane for spectrophotometric determination.

For diets containing algae, astaxanthin was analysed after lipids extraction (Folch *et al*. 1957) and quantified by spectrophotometry as described above. For diets containing synthetic astaxanthin the method of Schüep & Schierle (1995) was performed.

Due to the differences in amounts of astaxanthin in the test diets, data of astaxanthin concentrations in the serum were calculated as ratio (total blood astaxanthin per unit body weight to cumulative astaxanthin intake per unit body weight). A mean whole trout blood volume of 38 mL kg^{-1} at a water temperature of 16 °C was used for calculation according to Nikinmara *et al*. (1981). The results are given as mean ± SD. Data were subjected to analysis of variance and Duncan's multiple range test using the SAS-GLM procedure (SAS 1989). Statistical significances are indicated for $P < 0.05$ (Zar 1984).

Results

Total amount of carotenoid pigments of micro-algae *Haematococcus pluvialis* was 32 mg kg^{-1} on a dry weight basis of which keto-carotenoids accounted for 98.6%. Algal pigments are reported in Table 2. Astaxanthin monoester was the major carotenoid pigment of the algae and accounted for more than 80% of the total carotenoid concentration. Diets containing algae were formulated taking into account their astaxanthin profile. On this basis astaxanthin intake by fish was 0.32 ± 0.04 mg astaxanthin day^{-1} for trout fed algae supplemented diets and 0.37 ± 0.03 mg astaxanthin day^{-1} for trout fed synthetic astaxanthin supplemented diets.

Ratio (total blood astaxanthin per unit body weight to cumulative astaxanthin intake per unit body weight) in groups of rainbow trout fed the six test diets are shown in Table 3. After 5 days of feeding there was a significant effect of the pigment source on the ratio [total blood astaxanthin per unit body weight to cumulative astaxanthin intake per unit body weight]. Trout receiving synthetic astaxanthin showed a significantly ($P < 0.05$) higher ratio than trout fed algal astaxanthin. Increasing dietary bile extract did not lead to produce any effect on this ratio. Therefore, the interaction (pigment source × dietary bile concentration) showed no more effect.

Discussion

The astaxanthin content analysed in the algae is consistent with that reported in previous works (Grung *et al*. 1992; Lorenz & Cysewski 2000; Mendes-Pinto *et al*. 2001). Variations in carotenoid composition, especially mono and di-ester fractions, seem to be the result of different culture conditions and to depend on the age of the culture (Harker *et al*. 1996). However, this study confirmed that the major carotenoid pigment was astaxanthin in an amount as high as 98.6% of total carotenoids.

The micro-algae *Haematococcus pluvialis* occur in an encysted form surrounded by a thick cell wall, which may impede astaxanthin absorption by fish (Choubert & Henrich 1993). Therefore, an inefficient algae cell wall disruption leads to a limitation of its bioavailability (Sommer *et al*. 1991, 1992; Choubert & Henrich 1993). That is why algae

Table 2 Carotenoid pigments of the alga *Haematococcus pluvialis*

Carotenoid	% Total carotenoids DM[1]
Astaxanthin di-ester	17.2
Astaxanthin mono-ester	81.3
Astaxanthin free form	1.4

[1]DM: dry matter basis.

Dietary bile concentration (g kg^{-1})	BA/CAI algae astaxanthin[1]	BA/CAI synthetic astaxanthin[1]
0	1.74 ± 0.68^{b*}[2]	1.97 ± 0.67^{a*}
10	1.35 ± 0.54^{b*}	1.82 ± 0.34^{a*}
20	1.01 ± 0.41^{b*}	1.97 ± 0.74^{a*}
Significance	Mean square	*P*-values
Pigment source (PS)	4.6710	0.0004
Dietary bile concentration (BC)	0.7256	0.1226
Interaction (PS × BC)	0.6772	0.1403

[1] Data are mean ± SD.

[2] Within a row means with different superscript are significantly different, Duncan's multiple range test, $P < 0.05$. Within a column means with common asterisk are not significantly different, Duncan's multiple range test, $P < 0.05$.

Table 3 Changes in ratio: blood astaxanthin per unit body weight/ cumulative astaxanthin intake per unit body weight (BA/CAI) in groups ($n = 2$) of rainbow trout ($n = 10$) each fed the six test diets for 5 days

biomass was carefully ground before its addition to the diets. However this grinding step may explain the differences observed in the astaxanthin concentrations between the two series of test diets.

Fish were fed for 5 days to assure that astaxanthin serum concentration was maximum given the observation that a plateau of concentration is reached after one day of astaxanthin intake (Choubert *et al.* 1994). In these conditions mean serum astaxanthin concentrations can be used as an indicator of astaxanthin availability (Storebakken & Goswami 1996).

Despite the fact that there was a slight difference in amounts of astaxanthin in the test diets, our results showed that when no bile salts were added to the fish diet, serum astaxanthin concentrations of trout fed the diet supplemented with algae *Haematococcus pluvialis* were lower than those of trout fed the diet supplemented with synthetic astaxanthin. Algal astaxanthin is mostly in ester form (Renstrøm *et al.* 1981) while synthetic astaxanthin is in the free form (Klaüi & Bauernfeind 1981). Studies on absorption of ester/free forms of astaxanthin led to contradictory results: some authors claimed that the free form is better absorbed than the ester form (Foss *et al.* 1987; Storebakken *et al.* 1987; Choubert & Henrich 1993; White *et al.* 2002), some others reported that ester/free forms are equally absorbed (Barbosa *et al.* 1999; Bowen *et al.* 2002; White *et al.* 2002), lastly others observed that the ester form is better absorbed than the free form when the lipid level of the diet is low (Barbosa *et al.* 1999). From analogy with lutein recent data suggest that the bioavailability of ester form is not significantly different from that of free form (Bowen *et al.* 2002).

Blood astaxanthin concentration of trout fed diet supplemented with either algal or synthetic astaxanthin seemed not affected by an incremental increase of bile extracts in the diet. This was unexpected since it has been reported that bile extracts enhanced the absorption of β-carotene in rats (Gagnon & Dawson 1968) or human (Furr & Clark 1997). A possible biological explanation would be that bile concentrations used in this experiment were not sufficient. Based on the molecular weight of the glycodeoxycholic acid (449.6) the concentrations used were 2.427 and 4.855 mM for bile at a rate of 10 and 20 g kg^{-1}, respectively. In rat the uptake of β-carotene increased with increasing bile salt concentration up to 8–10 mM (El-Gorab *et al.* 1975). But another possible explanation would be the power of our experimental design. It is possible indeed that a real effect existed in our experiment but that no significant relationship was found because of large sampling variability or small sample size. Therefore, an *a posteriori* calculation was processed to evaluate the power of our analysis according to Searcy-Bernal (1994). Using from our analysis of variance $F = 2.18$, $k = 3$ and $n = 2$, a value of $f = 0.85$ was obtained and interpolation in the power table given by Searcy–Bernal yields a power of 0.19. Therefore, the design of our experiment had only 19% chance of detecting any differences. In this case it can only be stated that the results obtained in our experiment to show any effect of increasing dietary bile extracts on the blood astaxanthin concentration are not conclusive.

On these basis, more powerful experiments are therefore necessary to study if added bile extracts to fish diet can enhance blood astaxanthin concentration.

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